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Production of Vitamin B-12 in Tempeh, a Fermented Soybean Food†

IRENE T. H. LIEM, KEITH H. STEINKRAUS, AND TED C. CRONK††
Cornell University, Geneva, New York 14456

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Several varieties of soybeans contained generally less than 1 ng of vitamin B-12 per g. It was found that use of a lactic fermentation typical of tropical conditions during the initial soaking of the soybeans did not influence the vitamin B-12 content of the resulting tempeh. Pure tempeh molds obtained from different sources did not produce vitamin B-12. It was found that the major source of vitamin B-12 in commercial tempeh purchased in Toronto, Canada, was a bacterium that accompanies the mold during fermentation. Reinoculation of the pure bacterium onto dehulled, hydrated, and sterilized soybeans resulted in the production of 148 ng of vitamin B-12 per g. The presence of the mold, along with the bacterium, did not inhibit or enhance production of vitamin B-12. Nutritionally significant amounts of vitamin B-12 were also found in the Indonesian fermented food, ontjom.

Indonesian tempeh, a protein-rich vegetarian food, is one of the world's first meat analogs. Mycelia of molds belonging to *Rhizopus* overgrow hydrated, dehulled, and partially cooked soybeans, knitting them into a firm cake, which can be sliced and deep-fat fried or cut into cubes and used in place of meat in soups (4, 10, 12). In addition, it is one of the first vegetarian foods shown to contain nutritionally important amounts of vitamin B-12 essential for proper formation of erythrocytes and prevention of pernicious anemia (8, 13).

Indonesian fermented foods related to tempeh are ontjom (oncom) and bongkrek, made by fermenting, respectively, peanut and coconut presscakes with the mold *Neurospora sitophila* (9, 11, 13).

Foods derived from plants are thought to be devoid of vitamin B-12. Darken (1953) found low levels of vitamin B-12 in a few plants, but attributed the B-12 to contaminating microorganisms. Thus, soybeans would not be expected to contain vitamin B-12.

Generally, vitamin B-12 production is limited in nature to procaryotes. Bacteria of *Propionibacterium, Pseudomonas,* and *Clostridium* and of some of *Streptomyces* produce vitamin B-12 (6). Production of vitamin B-12 by molds is controversial. Generally, vitamin B-12 is not produced by eucaryotes. Nicholas (5) reported some production of vitamin B-12 by *Aspergillus niger.* No reports have been found of *Rhizopus* species producing vitamin B-12 or B-12-like activity.

Vitamin B-12 is essential in the human diet, although a minimum daily requirement has not been established. A recommended dietary allowance of 3 μg of B-12 per day has been set by the Committee on Dietary Allowances, 1974.

To determine the source of vitamin B-12 in tempeh, it was considered desirable to proceed in the following steps: (i) to confirm that commercial samples of tempeh do contain vitamin B-12; soybeans do not contain vitamin B-12, and pure tempeh molds do not produce vitamin B-12 during tempeh fermentation. (ii) To determine whether a bacterial acid fermentation during the initial soaking of soybeans (which occurs spontaneously in the tropics) might be responsible for B-12 production. (iii) To isolate other organisms, particularly bacteria, which might be present along with the mold in commercial tempeh and determine whether such organisms, if any, are responsible for B-12 production.

MATERIALS AND METHODS

Cultures. The microorganisms used in this study are listed in Table 1. Mold cultures were grown and stored on potato dextrose agar slants (Difco Laboratories, Detroit, Mich.). *Lactobacillus leichmannii* (ATCC 7830) stock culture used for microbiological assay was grown and stored on vitamin B-12 culture agar stabs (Difco). The unidentified bacterium was isolated as a single predominate species by streaking pulverized tempeh purchased in Toronto, Canada, on brain heart infusion (BHI) agar (Difco). The culture was then maintained on tryptone-glucose-yeast extract agar slants containing the following ingredients;

† New York State Agricultural Experiment Station journal paper 3061.
†† Present address: Pillsbury Mills Inc., Research Department, Minneapolis, Mn 55414.

TABLE 1. *Cultures used in the studies*

Organism	Source
Rhizopus species (Toronto)	Isolated from tempeh produced by Tjing Giok Tan, Toronto, Canada
Unidentified gram-negative rod-shaped bacterium (Toronto)	Isolated[a] from tempeh produced by Tjing Giok Tan, Toronto, Canada
Rhizopus species (Indonesia)	Isolated from dried, pulverized Indonesian tempeh brought to the United States by Bwee Hwa Yap (12)
L. leichmannii (ATCC 7830)	USDA, NRRL,[b] Peoria, Ill.
R. oligosporus (NRRL 2710)	USDA, NRRL,[b] Peoria, Ill.

[a] Bacterium (Toronto) was the single predominate bacterium that grew on the plates when pulverized, freeze-dried crude Toronto tempeh was streaked directly on BHI agar (Difco) and incubated at 37°C.

[b] USDA, NRRL, United States Department of Agriculture, Northern Regional Research Laboratory.

yeast extract (Difco), 3 g; tryptone (Difco), 5 g; D-glucose (Mallinckrodt Chemical Works, St. Louis, Mo.), 1 g; agar (Difco), 15 g; and distilled water, 1,000 ml. All cultures were grown at 37°C and maintained at 7°C between transfers.

Soybeans. Harosoy soybeans were used in all sample preparations, unless otherwise specified.

Preparation of tempeh and inoculum using petri dish cultures. Pyrex petri dishes (90 by 15 mm) were used as fermentation containers. Soybeans were soaked in distilled water for 2 h at 70°C. A 50-g portion of soaked soybean cotyledons dehulled by hand were added to each dish and autoclaved for 15 min at 121°C. The cooled, sterile soybean cotyledons were inoculated with the appropriate culture(s). For tempeh inocula, the appropriate pure mold as a slant culture on potato dextrose agar (Difco) or crude pulverized tempeh was used as inoculum. Tempeh fermentations were then incubated at 37°C for 18 to 20 h, at which time the soybean cotyledons had been completely overgrown with the white mold mycelia. For tempeh inocula, the tempehs were incubated for 24 h at 37°C, at which time black spores were formed.

All tempehs and tempeh inocula were then freeze-dried, pulverized in a sterilized Braun powder mill, and stored at 7°C. About 0.5 g of each freeze-dried, pulverized tempeh was extracted for its vitamin B-12 content, using the cyanide method of Skeggs (7), and assayed for vitamin B-12 content.

Vitamin B-12 assay. Vitamin B-12 content was determined by microbiological assay, using *L. leichmannii* (ATCC 7830) as the assay organism (1).

Tempeh samples assayed for vitamin B-12 content. Toronto tempeh was purchased frozen in a store in Toronto, Canada, and was freeze-dried. Indonesian tempeh was purchased in a market in Malang (East Java), cut into small pieces, sun-dried, and shipped by air to Cornell University, Ithaca, N. Y. Indonesian tempeh was bought from a food peddler in Malang (East Java), cut into small pieces, ovendried at 100°C for 1 h, and brought to the United States by the senior author. Tempeh made in California was received in frozen form and freeze-dried. All the dried samples were ground to a fine powder in a powder mill (Braun) and stored at 7°C until analyzed.

Tempehs produced with pure mold cultures. Petri dish cultures were inoculated individually with one of the following pure molds: *Rhizopus* species (Toronto), *Rhizopus* species (Indonesia), *Rhizopus oligosporus* (NRRL 2710), or uninoculated soybeans (control).

Tempehs produced using bacterial fermentation during soaking of soybeans. Soybeans for all samples were dried, dehulled, and pulverized. For sample 1 (control), soybeans were unfermented and unsoaked. For sample 2, soybeans were soaked for 15 h at 30°C (pH fell from 6.0 to 5.5). For sample 3, soybeans were soaked for 2 h at 70°C (no fermentation; no pH change). Soybeans in samples 2 and 3 were dehulled by hand, placed in petri dishes, autoclaved for 15 min at 121°C, cooled, and inoculated with *R. oligosporus* (NRRL 2710).

Tempeh produced with Toronto tempeh as a crude inoculum versus that with *Rhizopus* species (Toronto) as a pure inoculum. Petri dish cultures were inoculated individually with either 50 mg of freeze-dried, pulverized crude Toronto tempeh (sample 1) or 50 mg of freeze-dried, pulverized pure *Rhizopus* species (Toronto) tempeh (sample 2).

The isolated bacterium was inoculated into BHI broth (Difco) and incubated at 37°C for 13 to 15 h. Petri dish cultures were inoculated individually with one of the following: 1 ml of sterile BHI broth and 50 mg of pulverized pure *Rhizopus* species (Toronto) tempeh (control) (sample 1); 1 ml of the BHI culture of the isolated bacterium (sample 2); or 1 ml of BHI culture of the isolated bacterium and 50 mg of pulverized pure *Rhizopus* species (Toronto) tempeh (sample 3).

Tempehs produced with the isolated bacterium and varieties of soybeans. The varieties of soybeans used were Harosoy, Rampage (yellow or black seedcoat), and yellow or black soybeans from Indonesia (variety unknown). The isolated bacterium was inoculated into BHI broth and incubated at 37°C for 13 to 15 h. Each petri dish culture was inoculated with 1 ml of BHI culture of the isolated bacterium and 50 mg of pulverized pure *Rhizopus* species (Toronto) tempeh.

Tempeh produced by a pilot-plant method using the isolated bacterium to increase vitamin B-12 content. The method of tempeh production outlined by Steinkraus et al. (12) was used with some modification. The isolated bacterium was inoculated into BHI broth and incubated at 37°C for 13 to 15 h. Soybeans were soaked in distilled water for 2 h at 70°C and dehulled by hand. The soybean cotyledons were then cooked in acidified water (0.85% lactic acid) for 90 min. The water from cooking the soybeans was drained, and 50-g portions of the cooled cotyledons were placed in petri dishes, which were inoculated individually with one of the following: 50 mg of pulverized pure *Rhizopus* species (Toronto) tempeh or

50 mg of pulverized pure *Rhizopus* species (Toronto) tempeh and 1 ml of BHI culture of isolated bacterium. The samples were incubated at 37°C until the mold mycelium had knitted the beans into a tempeh cake.

Ontjom samples. Ontjom samples from Indonesia were obtained through the courtesy of A. G. Van Veen, Cornell University. Sample 1 was traditional ontjom made from peanut presscake and sample 2 was ontjom made from soybean residue. A 0.5-g portion of each dried, pulverized sample was extracted by the (7) cyanide method and assayed for its vitamin B-12 content.

RESULTS AND DISCUSSION

It was found that the major source of vitamin B-12 in commercial tempeh made in Toronto was a bacterium present as a single predominate species along with the mold during the fermentation. The Toronto tempeh sample was studied in greater detail since it contained the highest amount of vitamin B-12 (63.00 ng/g) among all the commercial samples assayed (Table 2). The 1974 Indonesian sample contained the lowest amount (4.00 ng/g). This latter figure correlates quite closely with the data reported by Steinkraus et al. (8), in which a value of 5 ng of vitamin B-12 per g was obtained from another Indonesian tempeh sample.

When the freeze-dried, pulverized Toronto tempeh was used as a crude inoculum for tempeh fermentation, an appreciable quantity of vitamin B-12 was produced (66.00 ng/g), as compared to the control tempeh (0.47 ng/g), in which pure *Rhizopus* species (Toronto) was used as inoculum (Table 3). When the bacterium from the Toronto tempeh was isolated and purified, reinoculation of this bacterium into sterile soybean substrate yielded a high vitamin B-12 content (148.00 ng/g, Table 4). The presence of the mold, together with the bacterium, did not significantly (by the *t* test at the 5% level of significance) enhance or inhibit the vitamin B-12 production. Apparently, this gram-negative, isolated bacterium is not sensitive to the antibacterial compound produced by the mold during the tempeh fermentation (14).

On the basis of the above evidence, it is possible that bacteria similar to the isolated bacterial species may be responsible for vitamin B-12 production in the Indonesian and California tempeh samples. The pure tempeh molds, whatever their source, did not produce nutritionally significant amounts of vitamin B-12 on the soybean substrate.

Soybeans contained so little vitamin B-12 activity that it would not be of nutritional significance. This finding (0.39 ng/g) correlates quite closely with data reported by Steinkraus et al. (8), in which the amount was found to be 0.15 ng/g. Soaking the soybeans either with or without an acid bacterial fermentation did not influence the vitamin B-12 content of the mold-fermented product (Table 5).

Although the amounts of vitamin B-12 produced by the bacterium on different varieties of soybeans (Harosoy, Rampage, Indonesian yellow, and Indonesian black) varied from 86.00 to 150.00 ng/g, there is no way of accounting for the differences unless they may be related to the cobalt concentrations in the beans. Vitamin B-12 requires about 4% of its molecular weight in the form of cobalt.

Steinkraus et al. (12) reported that the mold is the essential microorganism in tempeh fermentation, and the pure mold culture produced

TABLE 2. *Vitamin B-12 content of commercial tempehs made in Toronto, Indonesia, and California*

Sample no.	Source	Method of drying	Vitamin B-12 (ng/g)
1	Toronto	Freeze-dried	63.00 ± 2.00
2	Indonesia (1974)	Sun-dried	29.00 ± 5.00
3	Indonesia (1975)	Oven-dried	4.00 ± 1.00
4	California	Freeze-dried	15.00 ± 1.00

TABLE 3. *The effect of using Toronto tempeh (freeze-dried, pulverized) as a crude inoculum versus using Rhizopus species (Toronto) as a pure inoculum on vitamin B-12 content of tempeh*

Sample no.	Mold inoculum	Yield of vitamin B-12 (ng/g)
1	Toronto tempeh (crude inoculum)	66.00 ± 1.00
2	*Rhizopus* species (Toronto) (pure inoculum)	0.47 ± 0.04

TABLE 4. *Effect of isolated bacterium in producing vitamin B-12 during tempeh fermentation*

Sample no.	Inoculum	Yield of vitamin B-12 (ng/g)
1 (control)	1 ml of sterile BHI broth + pure *Rhizopus* species (Toronto)	0.47 ± 0.05
2	1 ml of BHI culture	148.00 ± 12.00
3	1 ml of BHI culture + pure *Rhizopus* species (Toronto)	150.00 ± 10.00

TABLE 5. *Effect of bacterial fermentation during soaking of soybeans on vitamin B-12 content of tempeh*

Sample no.	Method of soaking	Initial pH	Final pH	Yield of vitamin B-12 (ng/g)
1 (control)	None (dry beans)			0.00
2	70°C for 2 h	6.0	6.0	0.44 ± 0.08
3	30°C for 15 h	6.0	5.5	0.28 ± 0.08

TABLE 6. *Vitamin B-12 content of ontjom samples from Indonesia*

Sample no.	Substrate of ontjom	Yield of vitamin B-12 (ng/g)
1	Peanut presscake	31.00 ± 7.00
2	Soybean milk residue	23.00 ± 2.00

a tempeh organoleptically "better than tempeh produced in Indonesia," according to Indonesian students. But the findings in this study show that the presence of a certain bacterial species is very beneficial from the nutritional point of view. The presence of the bacterium, together with the mold during the tempeh fermentation, provides the consumer with vitamin B-12 that would not be available in tempeh from a pure mold culture fermentation. However, the presence of the bacterium also lengthens the fermentation time from 18 to 20 h to 25 to 30 h. The data for the vitamin B-12 level found in ontjom is listed in Table 6. The source of vitamin B-12 in this product is not known and needs further investigation. It would be interesting to determine whether vitamin B-12-producing bacteria are also present in ontjom.

The recommended dietary allowance of vitamin B-12 is 3 μg/day for adults (Committee on Dietary Allowances, 1974). When the concentration of B-12 is increased to 50 to 60 ng/g of tempeh, it becomes feasible for the consumer to get his or her daily requirement of vitamin B-12 by consumption of approximately 60 g, or about 2 ounces, of tempeh.

Studies on the identification of the gram-negative, rod-shaped bacterium are under way and will be reported later.

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